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19. ABSTRACT (Continue on reverse if necessary and identify by block number) We have developed a novel technique for rapidly studying microheterogeneity in a population of primate immunodeficiency viruses. Proviruses are rapidly cloned by using an integration - competent cytoplasmic extract from infected cells and integration - sensitive undersize cloning vectors based on bacteriophage lambda. The details of this cloning system are presented. This method yields many clones of integrated proviral DNA, each of which represents an independent clone of provirus contained in the original infected cell population. The utility of this vector to characterize <u>in vitro</u> integration products of a primate immunodeficiency virus (HIV-1) are illustrated as an example.					
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We have developed a retrotransposition-sensitive genetic vector that forms the basis for a comprehensive system for accelerated molecular characterization of retroviruses, and potentially, other transposable elements. It serves as a molecular cloning vehicle for retroviral proviruses, as well as an integration target for efficient "trapping" of intermediates of retroviral replication. Our vector is especially well-suited to the molecular cloning of HIV-I replication intermediates, as shown by our recent experiments, and promises to be a powerful tool for the molecular analysis of retroviruses in general.

In vitro integration assays -- a genetic assay¹ and a direct physical test² -- have been described previously. A genetic assay, from the Varmus laboratory, employs a λ gt WES vector that requires a suppressor for replication and plaque formation. Using a murine retrovirus genetically marked with a foreign tRNA suppressor, they recover pre-integration complexes from extracts of freshly infected cells and integrate the proviral DNAs into the concatemericized λ gt WES. The integrant clones are then selected by in vitro lambda packaging and plating on sup^o bacterial hosts. The Mizuuchi laboratory more recently reported a physical assay, that employed similar reaction chemistry, but used monomeric oX174 targets and gel-shift analysis to demonstrate the effect. Both of those assays, though very efficient, required prior knowledge of the identity of the retrovirus and of the restriction map or nucleotide sequences.

Inspired by an interest not only in the cloning of proviral integrants, but also in a species-independent, or "naive" way of molecular cloning of retroviruses, we created a vehicle that was integration-sensitive, but did not require genetic marking of the retrovirus, molecular probes, nor any prior knowledge of structural features. Such a target-vector was produced starting with a specialized phasmid construct, which could be cut and re-ligated in a single test tube, yielding extremely long, exclusively linear concatemers, comprised of undersized, 32Kb phasmid segments

as the repeating unit. Because this re-arranged vector would be subpackageable (in distinction from the full-length λ concatemers used in Ref.1) in λ in vitro packaging extracts, it should be at once strictly size-dependent and therefore integration-sensitive, requiring 6.5-20Kb of additional material for plaque formation (see Fig).

These properties were verified by replacing the oX174 target, used in the physical assays (in Mizuuchi's murine and our HIV-I experiments³) with our concatemeric, subpackageable target-vector. As predicted, the undersized phasmids captured the integration-competent proviruses with remarkable efficiency, recovering 30-100 complete genomes per 1ml of extract of infected cells. The resulting integrant/recombinants were isolated from the plaques without resorting to molecular probes. In fact, the identity of the retrovirus need not have been known. Thus, in principal, a previously uncharacterized retrovirus needs only to be within the acceptable size range (a 6.5-20Kb provirus), and should be replication-and integration-proficient.

In summary, significant features of our system are:

1. It is a cloning vector, as well as an integration target.
2. All integrant proviral clones are expected to be full-length and not to contain any host cellular flanking DNA.
3. Because most of the DNA non-essential to λ replication lies in the plasmid segment of the final target vector, integration events in the λ arms are counter-selected and many of the proviruses in the assay can therefore be recovered as plasmid recombinants after removal of the λ arms by excision with the appropriate restriction enzyme(s) (eg. Not I).
4. The assay is designed as a selection, rather than a screening; no molecular probes are necessary. Hence, as a "probe-less" strategy, it does not require previously having

molecularly cloned the virus, genetic marking or other manipulation of the virus, nor knowledge of the nucleotide sequence or restriction map: the retrovirus needs only to be ≥ 6.5 Kb in length and integration-proficient. Thus, molecular cloning and primary analysis of retroviruses can be accelerated by orders of magnitude and in a largely species-independent manner.

5. Potential commercial uses of the vector are cloning of biomedically important retrovirus, determination of retrotransposition activity for previously uncharacterized transposable elements, and screening for agents that block retroviral integration activity of human and animal pathogens such as HIV-1, HIV-2 and SIV.

References:

1. Brown, P.O., Bowerman, B., Varmus, H.E. and Bishop, J.M., (1987) Cell 49:347
2. Fujiwara, T. and Misuuchi, K. (1988) Cell 54:497
3. Farnet, C., and Haseltine, W.A. (1990) Proc. Natl. ACAD. Sci. (USA) (in press)
4. Hayashi, K., Nakazawa, M., Ishizaki, Y., and Obayashi, A. (1985) Nucleic Acids Res. 13:3261

METHODS

Construction of starting vector

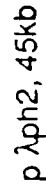
To construct p λ ph2, 2 μ g of λ GEMII (Promega) was cut with the restriction enzyme AvrII, 1 μ g of plasmid Bluescript Skm13 (Stratagene) cut with XbaI and XhoI, and 2 μ g λ FIXII (Stratagene) cut with XhoI. Each DNA was subjected to electrophoresis through a 1% Sea Plaque (FMC) low-melting agarose gel containing 1 μ g/ml ethidium bromide to separate the fragments: the λ GEMII left arm (20kb) and stuffer fragment (13kb), the plasmid (3kb), and the right arm of λ FIXII (9kb) were eluted from the gel bands, pooled, and sedimented. The DNA

pellet was resuspended in a 10 μ l reaction volume containing T4 DNA ligase. Ligations were incubated overnight at 13°C. 3 μ l of the ligation mix was packaged in Gigapack gold (Stratagene) and recombinant plaques were recovered on bacterial strain LE392. For this procedure, all enzymes were from New England Biolabs, approximately 5u. of each restriction enzyme and approximately 3 Weiss u. of T4 DNA ligase were used; and restriction digests, gel-elutions, ligation reactions and plaque recovery were performed strictly according to manufacturers' instructions.

Concatemerization:

p λ ph2 (25 μ g) was cut with EcoRI and HindIII in a 250 μ l reaction volume. The small linker fragments as well as the restriction enzymes were removed by selective precipitation in 2M ammonium acetate/2-volumes 2-propanol, followed by re-sedimenting with 0.3M sodium acetate/1-volume 2-propanol. The fragments were re-ligated using T4 DNA ligase, in a 200 μ l reaction volume containing 15% PEG-8000, for 0.5-2hr at room temperature. The DNA is then twice-precipitated, first in 2M ammonium acetate/2-propanol, then in 0.2M sodium chloride/2-propanol. After rinsing with 80% ethanol, the pellet was resuspended in 100 μ l of 10mM Tris, 1 mM EDTA pH 7.6, and any residual ligase was inactivated by heating to 68°C, 10min. The final product is highly concatemerized, and, due to the condensing agent PEG-8000, ligations are strictly inter-molecular⁴.

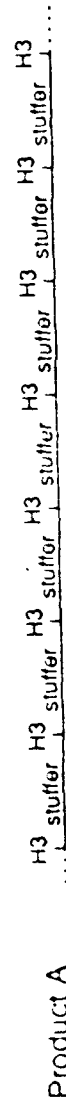
plasmid[®] Bluescript[®] SK M13-



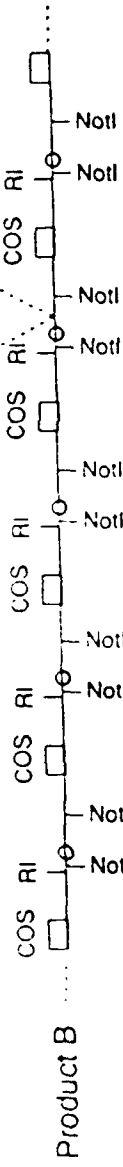
restriction - cut with Al, H3

selective precipitation in 2-propanol

re-ligation, T4 - DNA ligase



transposable element, 6.5 - 20 kb
(retrovirus, transposon, Ty element)

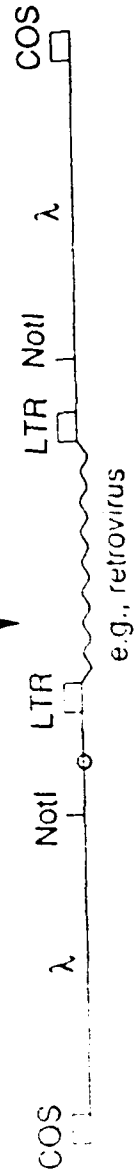


under-sized
(not packagable)
32 kb

full-length genome
(packagable)
38.5 - 52 kb

in vitro packaging

recovery plaques on bacterial lawns



Structures of the HIV provirus-target DNA junctions formed by integration in vitro:

- integration occurs at the conserved TG...CA dinucleotides at the viral DNA termini and generates a 5 base pair target site duplication.

1. AAAAA**TAAAC**TGGAAG . . . CTAGCA**TAAAC**AAATA
2. GAGGA**ATACC**TGGAAG . . . CTAGCA**ATACC**GATTC
3. TTGT**TCATG**TGGAAG . . . CTAGCA**TCATG**AGCGG

